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TITLE: In Vivo 18-FDG/18-Choline-Mediated Cerenkov Radiation Energy Transfer (CRET) Multiplexed Optical Imaging for Human Prostate Carcinoma Detection and Staging

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14. ABSTRACT:

Prostate cancer is treatable in its earliest stages, although treatment options for advanced forms are limited. Therefore, more sensitive means of early prostate cancer detection and new prostate cancer therapies are needed. Two novel biomarkers are proposed to associate with prostate cancer progression: the Thomsen-Friedenreich disaccharide (TF) antigen and the ErbB-2/ErbB-3 heterodimer (ErbB2/3). The objective of this proposal is to examine whether internal illumination via 18F-fluorocholine/18F-FDG Cerenkov radiation energy transfer (CRET) coupled with TF- and ErbB2/3- molecularly targeted near-infrared (NIR) QDs can be used to detect prostate cancer. We have shown that ErbB2/ErbB3 dimerization is heregulin mediated and upregulated in castrated mice bearing MDA-PCa-2b human prostate cancer xenografts. We have selected peptides from bacteriophage display libraries that target TF and ErbB2/ErbB3. The peptides have been attached to QDs and have been used to detect human prostate cancer cell lines that express TF, ErbB2/ErbB3. Biodistribution studies were performed in MDA-PCa-2b human prostate cancer castrated and uncastrated mice. The anti-TF and anti-ErbB3 QD could be visualized in MDA-PCa2b human prostate cancer xenografts using an ex vivo-in vivo CRET imaging protocol.

15. SUBJECT TERMS
androgen receptor (AR), Cerenkov radiation energy transfer (CRET), ErbB2, ErbB3, molecular imaging, PET, phage display, prostate cancer, quantum dots (QDs), Thomsen-Friedenreich disaccharide (TF)

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Table of Contents

Page

1. Introduction

Prostate cancer is treatable in its earliest stages, although treatment options for advanced forms are limited. Therefore, more sensitive means of early prostate cancer detection and new prostate cancer therapies are needed. Unquestionably, better biomarkers of prostate cancer would assist in diagnosis, predicting disease course, and therapy. Two novel biomarkers are proposed to associate with prostate cancer progression: the Thomsen-Friedenreich disaccharide (TF) antigen and the ErbB-2/ErbB-3 heterodimer (ErbB2/3). TF is a pan-carcinoma antigen expressed on 90% of carcinomas, including early and late stage prostate carcinomas. The ErbB2/3 heterodimer is thought to occur in ~85% of prostate cancers and is a biomarker for progression, aggressiveness, and recurrence in castration resistant prostate cancer. These biomarkers may serve as a foundation for new prostate cancer diagnostic imaging and therapeutic agents. Both molecular and functional imaging have gained attention as potential cancer diagnostics. Quantum dots (QDs) are being employed for in vivo molecular imaging because of their broad spectrum excitation, high fluorescence quantum yields, and large effective Stokes shifts; however, they are not ideal for use in vivo due to external visible light requirements and the resulting autofluorescence. Functional imaging utilizing positron emission tomography (PET) is currently in the clinic, and uptake of the PET tracers ^{18}F-fluorodeoxyglucose (FDG) and ^{18}F-fluorocholine in prostate tumors has been demonstrated. Unfortunately, distinction between benign and cancerous tissues with these PET tracers is not possible. A solution to this problem would be to couple QD based molecular imaging with ^{18}F-fluorocholine (or ^{18}F-FDG) radiation-luminescence (i.e. Cerenkov radiation) resulting from the ^{18}F positron emission as an internal source of illumination. The objective of this proposal is to examine whether internal illumination via ^{18}F-fluorocholine Cerenkov radiation energy transfer (CRET) coupled with TF- and ErbB2/3- molecularly targeted near-infrared (NIR) QDs can be used to detect prostate cancer. ^{18}F-fluorocholine PET imaging will be utilized to define the metabolically active tumor tissue, while molecularly targeted QDs will facilitate biomarker-specific diagnosis.

The specific aims of the proposal are to: 1) select peptides that target the ErbB2/3 heterodimer using novel parallel in vitro/in vivo phage display techniques; 2) generate NIR-QDs decorated with TF- and ErbB2/3-avid peptides for in vivo molecular targeting; and 3) employ multimodal, multiplexed in vivo imaging of choline uptake, and ErbB2/3- and TF- expression in various stages of prostate cancer in mouse models of human cancer.

2. Keywords: androgen receptor (AR), Cerenkov radiation energy transfer (CRET), ErbB2, ErbB3, molecular imaging, PET, phage display, prostate cancer, quantum dots (QDs), Thomsen-Friedenreich disaccharide (TF).

3. Overall Project Summary

Task 1. Parallel In Vitro Phage Display Selection and In Vivo Phage Display Selection.

Subtasks 1a through 1d were completed in year 1. Subtask 1e was completed in year 2.

Task 2. In Vitro Characterization of Selected Phage Clones and QDs Conjugated to Selected Peptides.

Subtasks 2a through 2g were completed in year 2.

Task 3. Optimize Components of CRET Imaging in the Prostate Carcinoma Tumor Model MDA-PCa-2b.

Subtask 3a: Inoculation and castration of nude mice to generate the MDA-PCa-2b human xenograft model of prostate carcinoma progression.

Progress: This subtask was accomplished in year 2. However, the rate of tumor take and growth of human prostate carcinoma MDA-PCa-2b was lower than expected (as reported in year 2 progress report). This subtask was repeated using different cell numbers. However, while the percent of mice that developed tumors increased, the length of time required for the tumor growth hindered experimental protocols. In addition, successful castration and development of castration resistant tumors was not improved. Consequently, tumors used within these studies were of various ages, sizes, and complexity. Thus our progress with in vivo studies has been hindered.

Subtask 3b: Optimize live ^{18}F-choline PET/CT imaging of nude mice bearing MDA-PCa-2b human prostate carcinoma xenograft model at 15 weeks post-castration (month 21).

Progress: This subtask was accomplished in year 2. We were unable to acquire or synthesize ^{18}F-choline in enough purity for these studies in year 3, thus, we attempted to utilize 18-FDG for imaging of MDA-PCa-2b human prostate carcinoma tumors. Unfortunately, the tumor uptake of 18-FDG by MDA-PCa-2b tumors was minimal.

Subtask 3c: Fluorescent optical imaging of molecularly targeted QDs in nude mice bearing MDA-PCa-2b human prostate carcinomas at 15 weeks post-castration.

Progress: This subtask was initiated within year 2 and completed in year 3. Tumor uptake of anti-TF, streptavidin coated QD with biotinylated p30-1 peptide, at 2 hours post-injection was imaged. However, the tumor to blood ratio was low. Thus further investigation, within year 3, of the pharmacokinetic profile of anti-TF, streptavidin coated QD with biotinylated p30-1 peptide, was performed in two non-castrated mice bearing MDA-PCa-2b tumors. The tumor uptake and retention of the molecularly targeted QD at 24 hours was not easily detectable within the images of the live mice (Figure 1). Ex vivo imaging of the excised tumors and organs revealed low tumor uptake and retention within only one of the tumors, which highlights the heterogeneity of this tumor model (Figure 2).

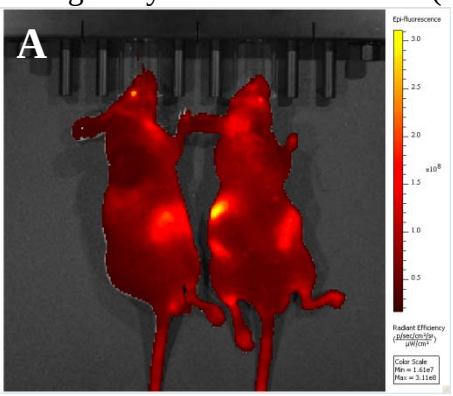
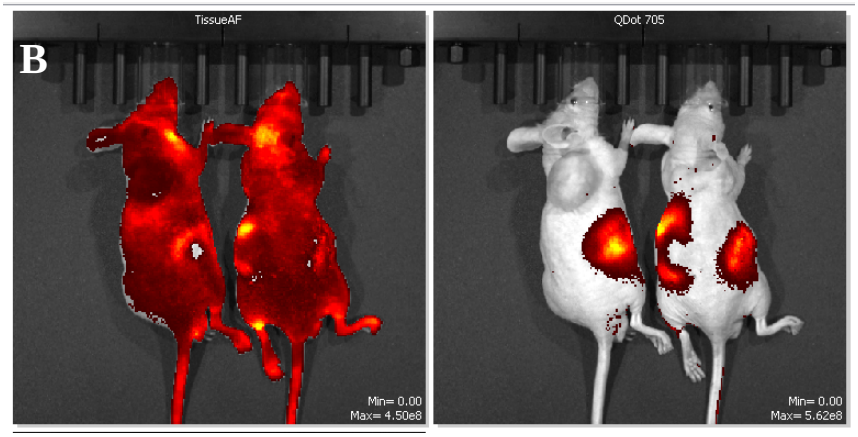


Figure 1: Optical Imaging of Molecularly Targeted p30-1/Qdot-705 within Intact Mice Bearing a Xenografted MDA-PCa-2b Human Prostate Carcinoma Tumors. Two non-castrated nude mice bearing MDA-PCa-2b human prostate carcinoma tumors received I.V. injections of 150 µL of 0.05 µM p30-1/SA-Qdot705. The animals were then (A) imaged 24 hours post-injection and (B) Living Image Software utilized to spectrally unmix the autofluorescence from the QD fluorescence.



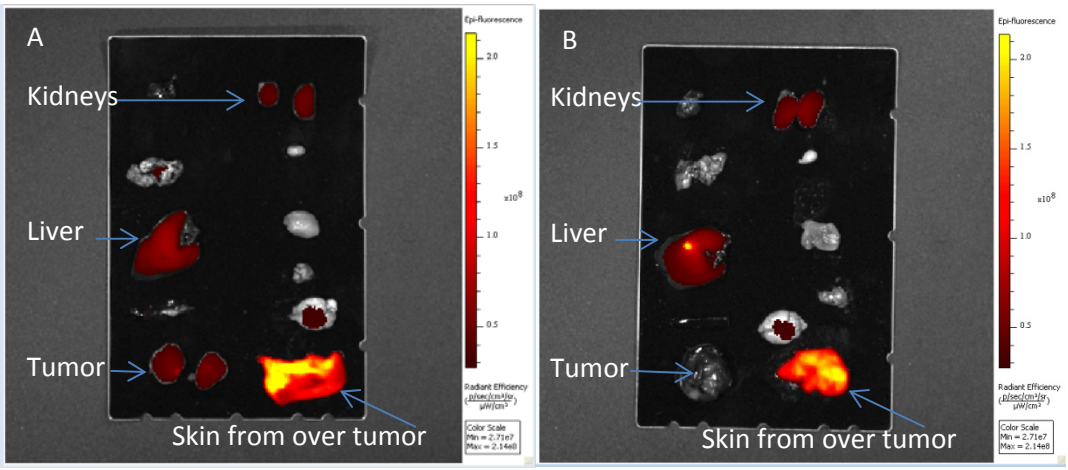


Figure 2: Ex Vivo Imaging of the Biodistribution of the Molecularly Targeted p30-1/SA-QD705 within Intact Mice Bearing a Xenografted MDA-PCa-2b Human Prostate Carcinoma Tumor. Two non-castrated nude mice bearing MDA-PCa-2b human prostate carcinoma tumors received I.V. injections of 150 µL of 0.05 µM p30-1/SA-Qdot705. The animals were then sacrificed and the tumors, organs, and tissues excised for ex vivo imaging.

It was hypothesized that the streptavidin coated QD could negatively affect the biodistribution of the p30-1/SA-QD705. One possible reason could be that the SA coated QD is much larger in diameter than the PEGylated QD, another possible explanation could be the surface charge of the SA proteins. Consequently, we purchased ITK Amino (PEG) QD (Invitrogen) for chemical coupling of peptides to QDs. These QDs are coated with amine-derivatized PEG. Consequently, we coupled the native carboxylic acid of the anti-TF and the anti-ErbB3 peptides to the amine-derivatized PEG using EDC. Unfortunately, this chemistry was not an option for the anti-ErbB2 peptide, KCCYSL, due the lysine within its sequence. Thus, this peptide was still used as a biotinylated peptide with an SA coated QD.

Subtask 3d: Pharmacokinetic analysis and comparative fluorescent imaging of molecularly targeted QDs in castrated and non-castrated nude mice bearing MDA-PCa-2b human prostate carcinoma xenografts.

Progress: Subcutaneous MDA-PCa-2b tumors were established in nude male mice. Of the mice inoculated, only 40% grew tumors at the expected rate. Once these tumors grew to 0.5 cm diameter one half of the mice were castrated. Following castration, as expected, the tumors regressed and regrew. These mice were then utilized for comparative optical imaging and further pharmacokinetic analysis of the new chemically coupled molecularly targeted QDs. Mice received tail vein injections of a mixed sample of QDs containing equal amounts of anti-ErbB3/QD605, anti-ErbB2/QD655, and anti-TF/QD705. Mice were then optically imaged, sacrificed, and the tumors and organs resected for ex vivo imaging and quantification of fluorescent signal (Figures 3 and 4).

Within the non-castrated mice, mouse #4 was utilized as a negative control. This mouse received QDs with no covalently attached molecularly targeting peptides. As expected there was no tumor uptake and only very low uptake within the clearance organs (Figure 3). Within the mice #1 through #3, which received molecularly targeted QDs, only mouse #3 had any tumor above background levels.

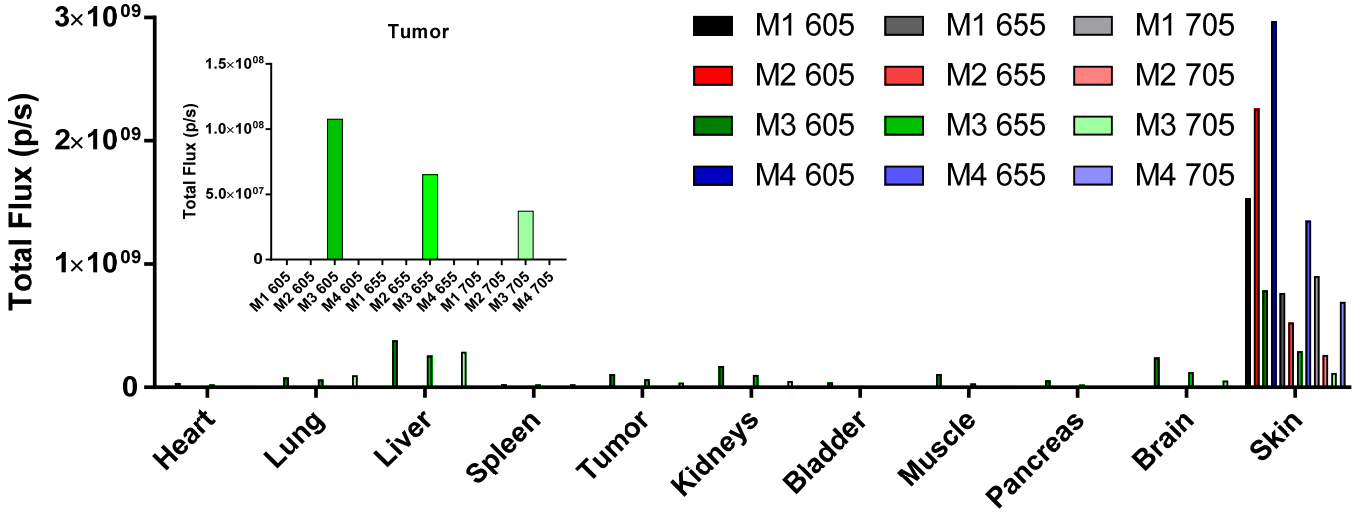


Figure 3: Ex Vivo Imaging of the Biodistribution of the Molecularly Targeted anti-ErbB3/QD605, anti-ErbB2/QD655, and anti-TF/QD705 within Non-Castrated Nude Mice Bearing MDA-PCa-2b Human Prostate Carcinoma Tumor Xenografts. Non-castrated mice bearing MDA-PCa-2b tumors #1 through #3 received tail vein injections of 100 µL of 0.5 µM mixed QD samples containing anti-ErbB3/QD605, anti-ErbB2/QD655, and anti-TF/QD705, while, mouse #4 received an equal mix of QDs with no molecular targeting peptides. The QDs were allowed to circulate for 24 hours before the mice were imaged, sacrificed, and organs and tissues resected for further imaging and quantification.

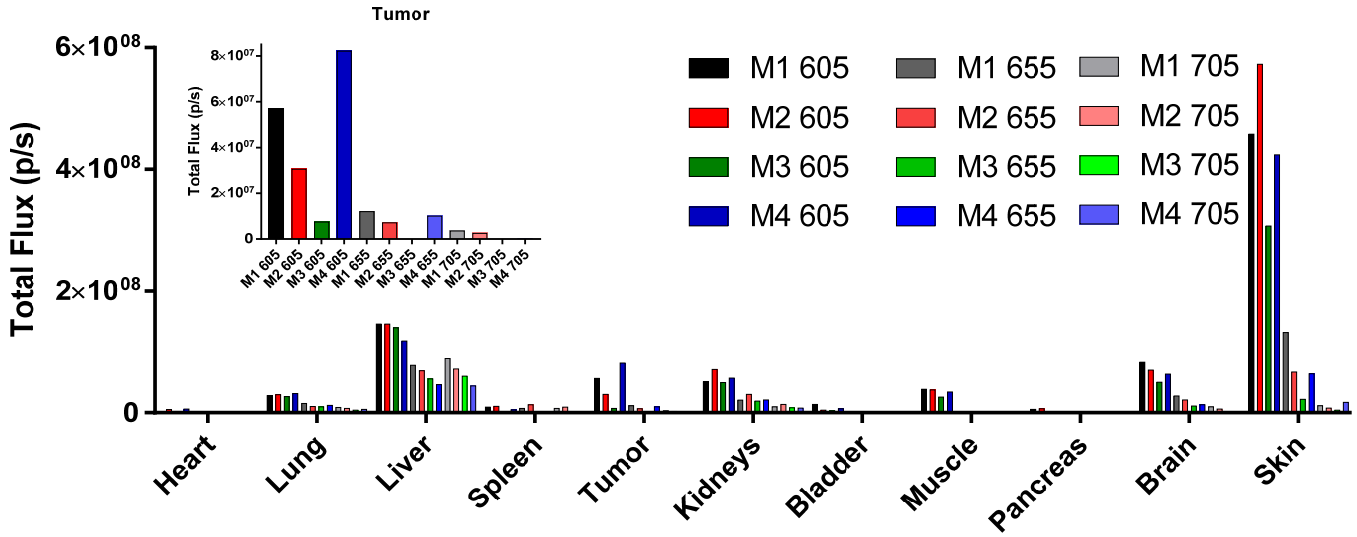


Figure 4: Ex Vivo Imaging of the Biodistribution of the Molecularly Targeted anti-ErbB3/QD605, anti-ErbB2/QD655, and anti-TF/QD705 within Castrated Nude Mice Bearing MDA-PCa-2b Human Prostate Carcinoma Tumor Xenografts. Castrated mice bearing MDA-PCa-2b tumors received tail vein injections of 100 µL of 0.5 µM mixed QD samples containing anti-ErbB3/QD605, anti-ErbB2/QD655, and anti-TF/QD705. The QDs were allowed to circulate for 24 hours before the mice were imaged, sacrificed, and organs and tissues resected for further imaging and quantification.

In comparison, the tumor uptake of the same mixed QD sample within castrated nude mice bearing MDA-PCa-2b hormone refractory tumors had significantly improved tumor uptake and retention of anti-ErbB3/QD605 (Figure 4). Anti-ErbB2/QD655 and anti-TF/QD705 tumor uptake and retention were also slightly improved when compared to the non-castrated tumor bearing mice. However, the lung and muscle uptake and retention was unexpected. Consequently, the tumor and select organs were collected, fixed in 10% buffered formalin, embedded in paraffin, and sliced for histological examination. After rehydration and epitope recovery the tumor tissues were blocked and probed for various biomarkers (Figures 5 and 6).

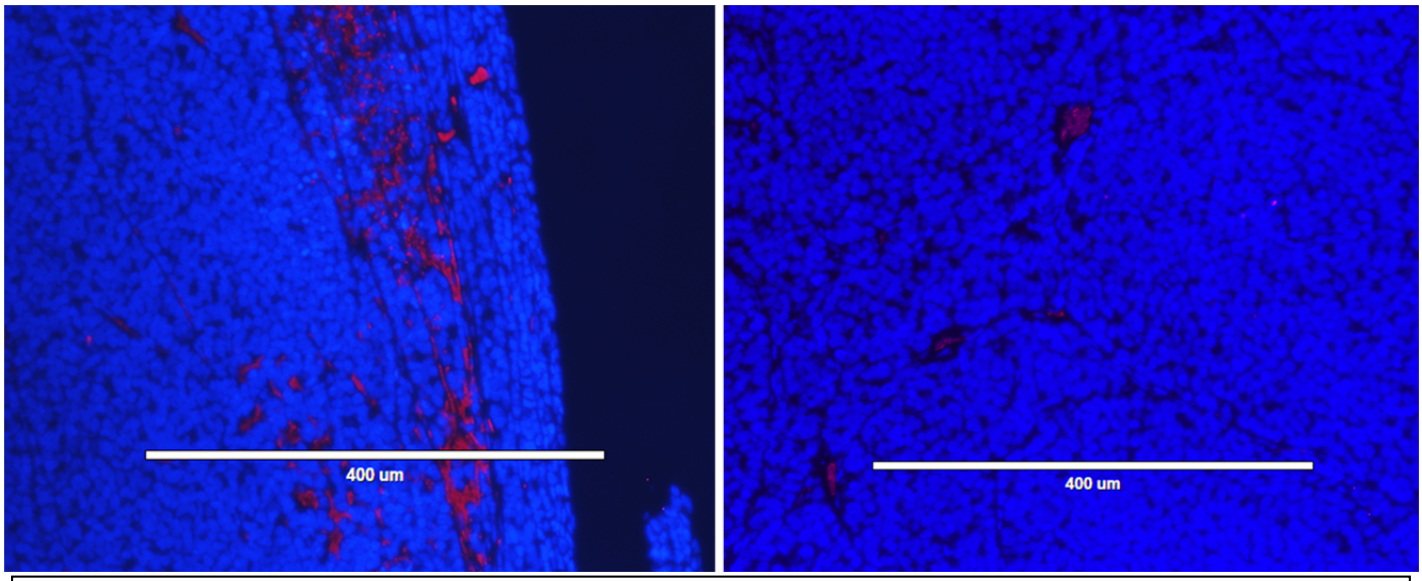


Figure 4: Microscopic Imaging of the Tissue Penetration of the Molecularly Targeted anti-ErbB3/QD605, anti-ErbB2/QD655, and anti-TF/QD705 within MDA-PCa-2b Human Prostate Carcinoma Tumor Xenografts. Castrated mice bearing MDA-PCa-2b tumors which received tail vein injections of mixed QD samples were sacrificed 24 hours post-injection and organs and tissues resected for further processing and analysis. Here, FFPE tumor tissue was de-paraffinized, re-hydrated, and submitted to epitope recovery prior to blocking and DAPI staining.

Initial histological investigation into the tumor uptake and retention of the QDs was performed using 5 micron thick slices of tumor tissue which was counter stained with DAPI to identify the nuclei of cells and help delineate areas of tissue from vasculature and interstitial spaces. Figure 4 shows the vast majority of the QDs are contained within the vasculature and interstitial spaces with only 3 or 4 potential points of QD fluorescent signal within the actual tumor tissue. This data shows that the QDs are unable to extravasate the vasculature. This taken in combination with the lung and muscle uptake is suggestive of aggregation of the QDs.

Further exploration into the biomarkers revealed heterogeneity within each tumor type (ie. hormone sensitive versus hormone refractory). For example, the androgen receptor (AR) was expected to be present in hormone sensitive tumors from non-castrated mice and absent in hormone refractory tumors from castrated mice. However, the levels of AR found within non-castrated mice #2, #3 and #4 were surprisingly low in comparison to non-castrated mouse #1. Additionally, castrated mice #3 and #4 seemed to retain an unexpected amount of AR even after 15 weeks post-castration. Similar issues were found with ErbB3 levels and TF levels (PNA staining). Interestingly, heterogeneity was also found within individual tumors as well (data not shown). But importantly, over all, the hormone refractory tumors resected from the castrated mice possessed higher levels of the probed biomarkers. This data is in agreement with the ex vivo imaging analysis of the biodistribution of the molecularly targeted QDs.

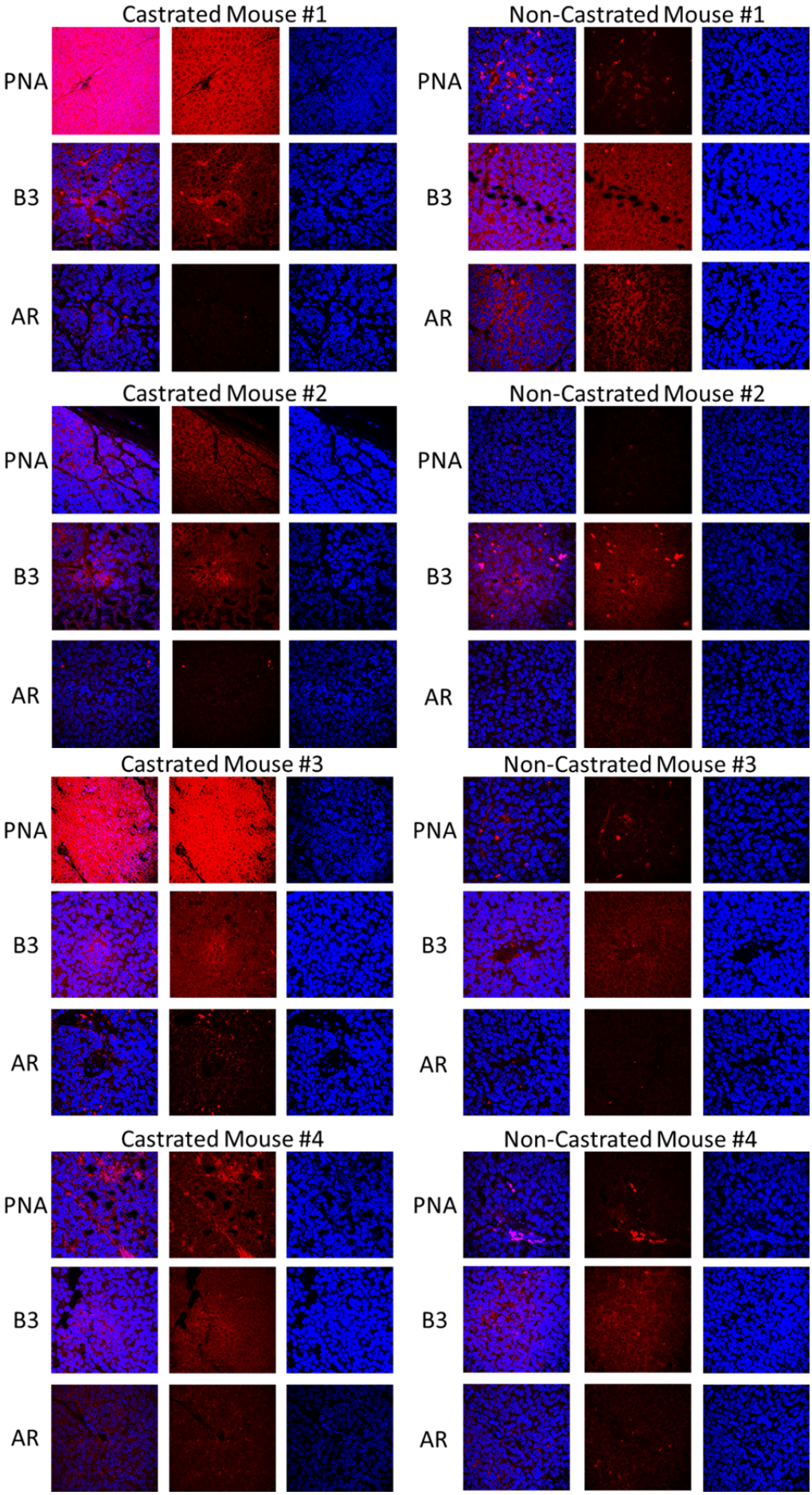


Figure 5: Microscopic Imaging of Biomarkers within Excised MDA-PCa-2b Tumors from Non-Castrated and Castrated Nude Mice. Castrated and non-castrated mice bearing MDA-PCa-2b tumors which received tail vein injections of mixed QD samples were sacrificed 24 hours post-injection and organs and tissues resected for further processing and analysis. Here, FFPE tumor tissue was de-paraffinized, re-hydrated, and submitted to epitope recovery prior to blocking, biomarker staining, and DAPI staining.

Task 4. Standardize Protocol for In Vivo Multiplexed CRET Imaging of Nude Mice (Castrated and Intact) Bearing MDA-PCa-2b Human Prostate Carcinoma Xenografts.

Subtask 4a: Inoculation and castration of 9 nude mice to generate the MDA-PCa-2b human xenograft model of prostate carcinoma progression (month 24)

Progress: We inoculated a nude mice with MDA-PCa-2b human prostate carcinoma cells and ananlyzed CRET imaging in years 2 and 3. Data is presented below.

Subtask 4b: Standardize CRET multiplexed optical imaging in nude mice (intact, 1 week post-castration, and 15 weeks post-castration) bearing MDA-PCa-2b human prostate carcinoma xenografts (months 28-29)

Progress: The first attempt at in vivo CRET imaging was performed within a nude mouse with no tumor (data not shown).

The second attempt at in vivo CRET imaging was performed within three nude mice with MDA-PCa-2b tumors (Figure 6). The mice were fasted overnight and placed in a warm and dark environment to prevent brown fat uptake. Each mouse then received an I.P. injection of 18-FDG followed immediately by intra-tumoral injection of molecularly targeted p30-1/Qdot. Tumor 1 received 0.8 µM, Tumor 2 received 0.08 µM, and Tumor 3 received 0.008 µM intra-tumoral injection. After 30 minutes the mice were sacrificed and tumors harvested. Fluorescent imaging revealed that the molecularly targeted p30-1/Qdot was still localized within the tumor. However, each tumor had varying levels of 18-FDG uptake and retention 1, 1.3, and 0.7 µCi, were measured in Tumors 1, 2, and 3, respectively.

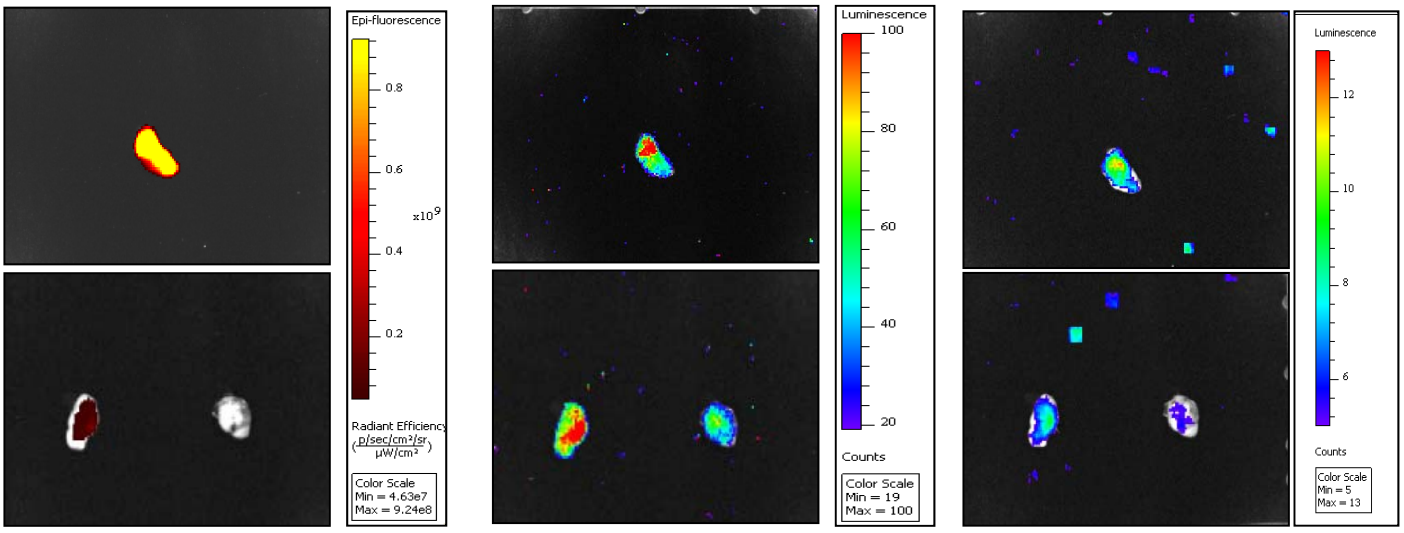


Figure 6: Optical Imaging of p30-1/Qdot-705 CRET Signal within MDA-PCa-2b Tumors. Three fasted nude mice were placed in a warm and dark environment for 30 minutes prior to receiving an I.P. injection of 300 µCi of 18-FDG followed immediately by intra-tumoral injections of, 0.8 µM, 0.08 µM, or 0.008 µM p30-1/Qdot. The mice were kept in the warm and dark environment for another 30 minutes, followed sacrifice, tumor harvest, and optical imaging: fluorescent imaging (A), luminescent Cerenkov imaging (B) and CRET imaging (C).

Task 5. Evaluate Diagnostic/Staging Potential of Multiplexed CRET Imaging within TRAMP Mice.
Evaluate Diagnostic/Staging Potential of Multiplexed CRET Imaging within TRAMP Mice (months 30-36).

Subtask 5a: TRAMP mice (ages 8, 12, 16, or 20 weeks old) with and without castrations will be imaged utilizing the standardized multiplexed CRET imaging protocol (30 mice) (months 31 – 36).

Progress: We are attempting to establish enough TRAMP mice to perform these studies and are still exploring CRET imaging potential in castrated and non-castrated MDA-PCa-2b human prostate carcinoma xenografts.

Subtask 5b: Histopathologic analysis of imaged TRAMP mice to validate diagnosis of stage of tumor development and/or progression (months 31-36).

Progress: To be completed year 3 and in 1 year no-cost extension.

4. Key Research Accomplishments

- Synthesize the TF, ErbB2 and ErbB3 avid peptides with a biotin for coupling to streptavidin coated QDs.
- Synthesize the TF, ErbB2 and ErbB3 avid peptides with no biotin for chemical coupling to amine derivatized PEG coated QDs.
- Characterize binding of TF, ErbB2 and ErbB3 avid PEG-QDs to human prostate MDA-PCa-2b carcinoma cells using fluorescent imaging.
- Compare biodistribution of molecularly targeted SA-QD versus PEG-QD.
- Compare biodistribution of molecularly targeted QDs within hormone sensitive and hormone refractory MDA-PCa-2b tumor bearing mice.
- Microscopically analyze QD tumor penetration and biomarker expression of the MDA-PCa-2b tumors.

5. Conclusions

We have shown that biomarker status is indeed changed, as predicted, within hormone refractory MDA-PCa-2b tumors as compared to hormone sensitive MDA-PCa-2b tumors. However, the cancer biology/ biochemistry are more complex and heterogeneous than originally hypothesized. This finding resulted in possible QD aggregation, lack of QD extravasation/tissue penetration, and unpredictable tumor take and tumor growth rates. The anti-TF and anti-ErbB3 QD could be visualized in MDA-PCa2b human prostate cancer xenografts using an ex vivo-in vivo CRET imaging protocol. The anti-TF QD gave the most positive results. We hope to optimize tumor take and uptake rates for remainder of grant period and couple peptides to a smaller framework for better optimization.

6. Publications, Abstracts, Presentations. Nothing to report

7. Inventions, Patents, Licenses. Nothing to report

8. Reportable Outcomes.

- CRET imaging has been developed and used with marginal success in molecular imaging.
- TF, ErbB2 and ErbB3 avid peptide-based QDs have been generated and can be used for prostate cancer detection in vitro.

9. Other Achievements. Nothing to report

10. References

11. Appendices. None